LYTIC EFFECTS OF SPERM EXTRACTS ON THE EGGS OF MYTILUS EDULIS[1]

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Introduction

A variety of specific substances are obtainable from the eggs and sperm of animals (reviewed by Tyler, 1948, and Bielig and von Medem, 1949). From the sperm may be obtained antifertilizins and various lytic substances such as hyaluronidase from mammalian sperm, an egg surface lysin from sea-urchin sperm, and membrane lysins from the sperm of various animals.

As described in a preliminary note (Berg, 1949), sperm extracts of *Mytilus edulis* are capable of dissolving or preventing the formation of the cement which normally binds the segmentation blastomeres together. The present report concerns the characteristics of the active agent particularly with respect to its chemical nature and relationship to an egg membrane lysin which is also present in the sperm extracts. The cement dissolving activity of the extracts is closely associated with the membrane lysin; however there is evidence which suggests that the effect is brought about by a separate substance.

Lysis of jelly coats and membranes of eggs by sperm extracts has been described by previous investigators. Hibbard (1928) and Wintrebert (1929, 1933) discovered that strong sperm suspensions or sperm extracts of the amphibian, *Discoglossus*, caused dissolution of an egg coat. Tyler (1939) extracted membrane lysins from the sperm of the keyhole limpet, *Megathura crenulata*, and the abalone, *Haliotis crackerodii*, which dissolved the membranes of the eggs. von Medem (1942, 1945) subsequently described similar membrane lysins in other species of limpets and abalones. Ruffo and Monroy (1947) reported the dissolution of the egg coats of several species of sea-urchins by a hyaluronidase-like substance obtainable from the sperm, and Monroy (1948) later reported the presence of a membrane lysin in the sperm of *Pomatoceros*.

The action of the above membrane lysins on cleavage of the eggs was in some instances not reported or claimed to have no effect.

Methods

The gametes of the bay mussel, *Mytilus edulis*, were used for the majority of these experiments, although at times the testes of the larger species, *Mytilus californianus*, were used for preparing considerable quantities of sperm extracts. Animals, collected from the San Francisco Bay or the open coast, were kept at 5° C.

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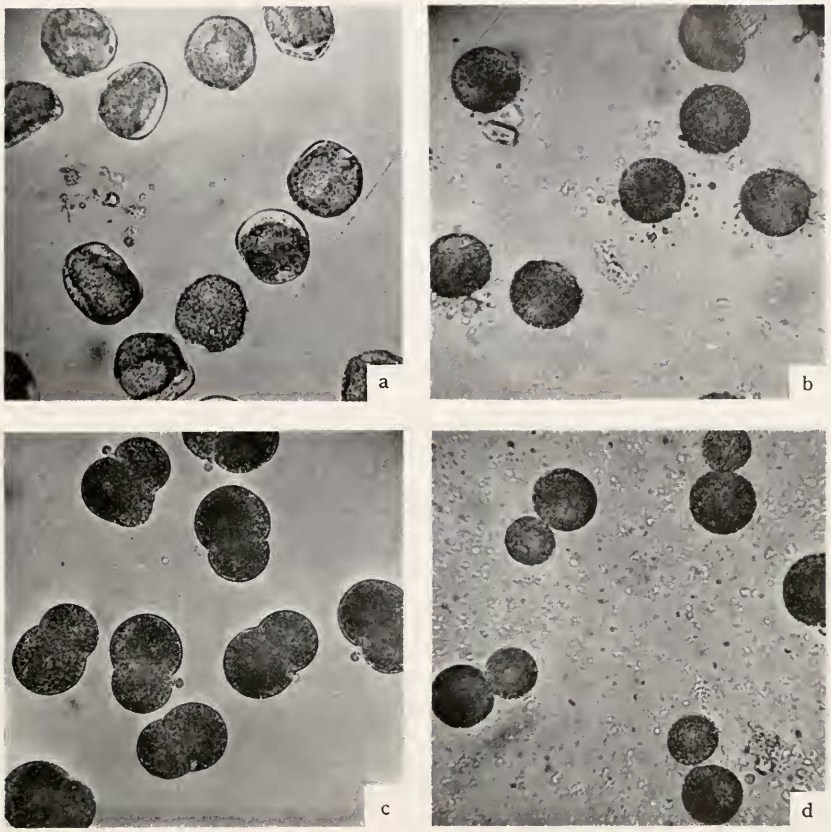
PLATE 1



Effects of *Mytilus edulis* sperm extracts on the egg membrane and intercellular cement: a, membranes rendered visible by plasmolysis of eggs with hypertonic sea water; b, dissolution of membranes after two minutes treatment with a sperm extract; c, normal appearance of eggs after first cleavage; d, cleavage of eggs in a sperm extract.

for as long as three or four days. Viable eggs and sperm were obtained by allowing the animals to spawn in individual finger bowls of sea water at room temperature.

Sperm suspensions, used for the preparation of sperm extracts, were obtained either by concentrating naturally spawned sperm by centrifugation or by the shedding of sperm from a number of excised testes in a small volume of sea water. The latter procedure proved to be particularly convenient for obtaining very dense suspensions of sperm. Sperm extracts were prepared by freezing a sperm suspension followed by thawing and grinding the frozen mass in a mortar. This resulted in a viscous liquid which on centrifugation separated into a white precipitate and a straw colored supernatant. The latter, representing a crude sperm extract, contained the egg membrane lysin and cement dissolving factor.

Ripe eggs of *M. edulis*, as obtained by spawning, are about 63 microns in diameter, somewhat opaque, and irregularly shaped. There is no egg coat or jelly layer

although a thin membrane can be made visible by cytolysis of the egg in distilled water or by plasmolysis in hypertonic sea water. The latter treatment was used as a standard method for revealing the membranes for subsequent tests in sperm extracts. Sea water concentrated to nearly twice the normal strength was used as a plasmolytic agent, and after 10–20 minutes exposure to this solution the membranes became clearly visible (Plate 1a).

The cement dissolving activity of a sperm extract was determined by whether the first cleavage blastomeres became noncohesive and spherical. Since the membranes themselves act as physical hindrances to separation or rounding up of the blastomeres, they were routinely removed from the eggs shortly after fertilization. As a standard procedure the membranes were removed by a strong sperm extract 10–20 minutes after fertilization of the eggs. The "naked" eggs were transferred through several dishes of sea water to remove all traces of the sperm extract and then added to the test sperm extracts 10–15 minutes before the first cleavage.

Effects of Sperm Extract on Egg Membranes and Intercellular Cement

The presence of an egg membrane lysin in *M. edulis* sperm extracts is easily demonstrated. An extract prepared by the method of freezing and thawing will cause the disintegration of the membranes in a few seconds or minutes depending upon its strength. The dissolution begins by a thinning and buckling of the membrane. Gradually the sharp edges become less distinct until only a residue remains as a corona about the egg surface. In all except weak sperm extracts, this corona eventually disappears. Plate 1a shows the appearance of the membranes after plasmolysis of the eggs and 1b shows eggs of the same sample two minutes after treatment with a sperm extract.

The preceding plasmolytic treatment necessary to expose the membranes has no effect on the lytic process since the membranes can be dissolved as readily from unplasmolyzed eggs.

Sperm extracts, in addition to dissolving the membranes, also have a striking effect on the early cleavages of the eggs. Cleavage of *M. edulis* eggs has been described and pictured in detail by Field (1922) and only a brief account need be given here. Shortly after fertilization the eggs lose their irregular shapes and by the time the first polar body is formed they are spherical. Shortly after the second polar body is formed the egg surface in the vegetal hemisphere becomes wrinkled, followed by a bulging of the cytoplasm in this area. The cytoplasmic bulge, or polar lobe, causes the egg to assume a pear shape (Fig. 1a) and as the lobe increases in size the egg becomes flattened and furrowed in the animal hemisphere (Fig. 1b). This gives the illusion that the egg has divided into three cells, the so-called trefoil stage. The first cleavage plane, beginning at the animal pole, curves to one side in the vegetal hemisphere, so that the polar lobe is separated from the AB cell, but retains connection with the CD cell (Fig. 1b). Fusion of the polar lobe with the CD cell occurs soon after the trefoil stage resulting finally in an unequal cleavage. The first cleavage blastomeres are strongly cohesive and there is a considerable flattening of their area of contact (Fig. 1c, Plate 1c).

If eggs are allowed to cleave in a sperm extract, the appearance of the polar lobe and blastomeres is quite different from the normal pattern. The polar lobe appears

as a bulge in the vegetal hemisphere, as in the controls, and the eggs assume a pear shape (Fig. 1d). However, shortly afterwards, at what would correspond to the trefoil stage, the appearance diverges from that of the controls. At this stage (Fig. 1e) the polar lobe extends directly out from the eggs as a globule of cytoplasm connected to the CD cell by a stalk. The plane of cleavage is straight instead of curved and as the furrow advances across the cytoplasm the newly formed edges become separated instead of cohering as in the controls. After cleavage the polar lobe is retracted into the CD cell, although occasionally it may be pinched off completely. At the close of first cleavage, the blastomeres are spherical and may be either completely separated or barely touching (Fig. 1f, Plate 1d). In a weak sperm extract the blastomeres remain attached, but the area of contact is noticeably reduced as compared to the controls.

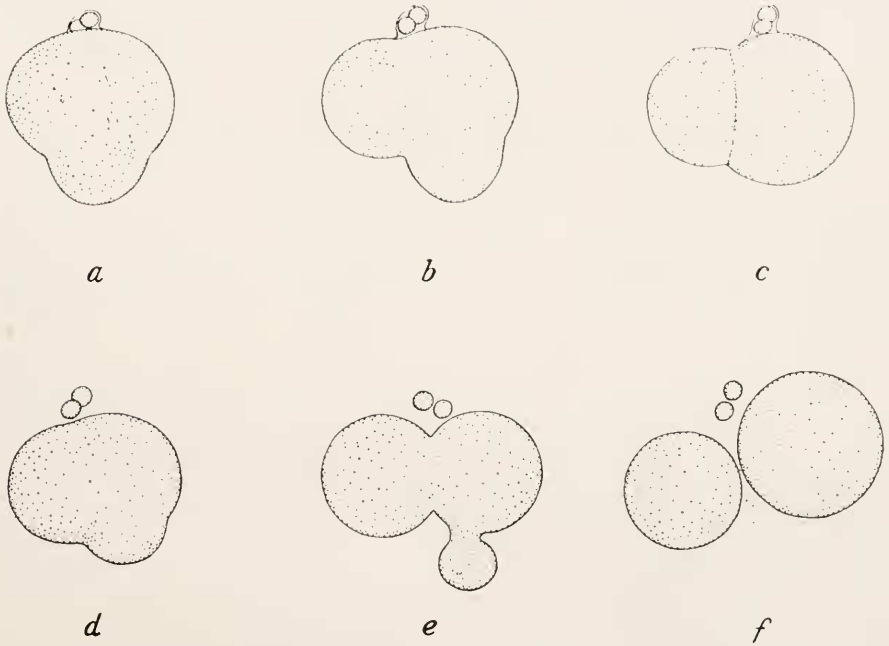


FIGURE 1. Normal first cleavage of *Mytilus edulis* eggs (a-c) and cleavage in a sperm extract (d-f) : a, formation of polar lobe; b, trefoil stage; c, completion of first cleavage; d-f, corresponding stages of cleavage in a sperm extract.

The cement dissolving activity of sperm extracts is maximal during the process of cleavage. Eggs which have already completed the first cleavage are affected very little. However, if they are allowed to remain in the extract, the A, B, C and D cells, formed by the second cleavage, become noncoherent and assume a spherical shape. Embryos which are reared in sperm extracts usually develop into a loose mass of ciliated cells.

Essentially the same pattern of cleavage and development is obtained if the eggs are allowed to cleave in calcium free sea water. Although there are slight differences in appearance of eggs cleaving in a sperm extract as compared to those in calcium free sea water, it seems reasonable to assume that the sperm extract acts

in a similar manner as lack of calcium—by dissolving or preventing the formation of the cement which holds the cells together.

Fertilized eggs which have had the membranes removed, often become sticky and adhere to the bottom of the containers. Addition of a sperm extract causes the adhesiveness to disappear which supports the view that the extract contains a substance capable of dissolving the cement. This, in the broad sense of the term, may be considered a lytic activity of the sperm extracts and the active agent will be referred to tentatively as the cement lysin.

Properties and Relationship of Active Agents in the Sperm Extracts

A lytic action of sperm extracts on the intercellular cement has not been described before, either in *Mytilus* or other forms, and it seemed worth-while to investigate the causative agent in regard to its occurrence, identity or non-identity with the membrane lysin, and some of its chemical properties.

Table 1

Bioassays of the lytic activities of various types of sperm extracts.
Each figure represents a separate experiment.

	Time for membrane dissolution of minutes	Titer of cement lysin
Concentrated sperm suspensions	4, 6	16, 32
Supernatants of above suspensions	Slight effect after 45 min. to 1 hour	4, 8, 8
Extraction by freezing and thawing	2, 5, 3	128, 64, 64
Extraction by heat	No effect	16, 16
Acid extraction	5, 5	32, 64
Alkaline extraction	15	16

In many of the experiments to be described, it was necessary to use bioassays of the sperm extracts. The time interval for dissolution of the egg membrane is a satisfactory assay for the membrane lysin; however, there is no such convenient time factor involved in the dissolution of the cement. Accordingly a dilution assay was employed in which the sperm extract was diluted ½, ¼, ⅛, etc., and the greatest dilution at which a perceptible effect occurred indicated the strength of the original sperm extract. The strength or titer of the extract was most conveniently expressed as the reciprocal of the greatest dilution exhibiting an effect. A strong sperm extract may have a cement dissolving titer as high as 128; accordingly there is a considerable range for comparison of different extracts. Using these bioassays, the strengths of the effective agents were determined in sperm suspensions, their supernatants, and in extracts prepared by various methods. Results of typical experiments are summarized in Table 1.

Dilute sperm suspensions (as obtained by spawning) and their supernatants had no perceptible effect on the egg membranes or early cleavages. Sperm suspensions, roughly about fifty times as concentrated as above, were prepared by the shedding of the sperm from excised testes. These suspensions dissolved the membranes about as rapidly as extracts prepared from the same suspensions, but the cement dissolving titers were proportionately weak as compared to the extracts (Table 1). This difference, however, seems too slight to be interpreted as any real separation of lytic activities. The supernatants, as obtained by centrifugation, caused dissolution of the intercellular cement, but had an extremely weak lytic effect on the mem-

branes. This was originally interpreted as a separation of active agents; however, assays of the supernatants showed that the relative strengths were comparable to those normally found in an extract. Thus one-eighth dilutions of extracts prepared by freezing and thawing resulted in membrane-lytic strengths equivalent to that of the above supernatants and cement dissolving titers from 4 to 8 which are also equivalent to that of the supernatants.

Dissolution of the membranes and intercellular cement is brought about by sperm extracts prepared by acidification or alkalization. A portion of a concentrated sperm suspension was brought to pH 1.5 by addition of HCl, centrifuged and the supernatant neutralized. The supernatant had nearly the same activity (Table 1) as a comparable extract prepared by freezing and thawing. Considerably weaker extracts were obtained by alkalization (pH 10 or above); however, the relative strengths of the lytic activities were the same as by other methods of extraction.

Sperm suspensions after heating to 100° C. for a few minutes yielded supernatants which dissolved the intercellular cement, but lacked the membrane lysin; however, this was not a differential extraction, since, as will be pointed out later, the membrane lysin is destroyed by heat.

It became evident that the lytic activities of sperm extracts were very closely associated and could not be separated readily by these methods into two specific fractions. Accordingly, further tests were carried out to determine some of the chemical properties of the active agents in the hope that these tests might lead to conclusions regarding their relationship.

The active agents of the sperm extracts are evidently large molecules as shown by their non-dialyzability. In a typical experiment 50 cc. of sperm extract were dialyzed in a cellophane tube for two days in an equal volume of sea water. Tests of the dialysate on the egg membranes and early cleavages were negative, whereas the solution inside the tube retained nearly the original strength.

Precipitates obtained by 50 per cent to 90 per cent saturation with $(NH_4)_2SO_4$ or by dialysis against distilled water contain the active factors. Precipitation by $(NH_4)_2SO_4$ was brought about by slowly adding at 3° C. a saturated solution of the salt to the sperm extract until the desired concentration was reached. At 50 per cent saturation a floccular white precipitate usually formed within a few hours. Since centrifuging tended to disperse rather than to concentrate the precipitate, it was allowed to settle out for a period of 24 hours and the supernatant decanted. This did not give complete separation, but was sufficient to show that the bulk of the activity of the sperm extract was in the precipitate. Table 2 records assays of the lytic activities of the precipitate and supernatant. The residual activity of the supernatant was probably due to incomplete separation of the precipitate and perhaps also to incomplete precipitation of the active substances.

A heavy and complete precipitation of the active factors was obtained at 90 per cent saturation with $(NH_4)_2SO_4$ (Table 2). Concentrations intermediate between 50 per cent and 90 per cent gave results similar to a 50 per cent saturation. No differential precipitation of active agents occurred.

Dialysis against distilled water also brings about precipitation of the active substances from a sperm extract. In a typical experiment, 50 cc. of sperm extract were dialyzed against 4 liters of distilled water at 3° C. Dialysis was carried out for 48 hours although a precipitate began to appear within several hours. Assays

TABLE 2

Precipitation of lytic activities of a sperm extract

Treatment of sperm extract	Time for dissolution of egg membranes		Titer of cement lysin
50% saturation with $(NH_4)_2SO_4$	supernatant	15 minutes	4
	precipitate	1/2 minutes	64
90% saturation with $(NH_4)_2SO_4$	supernatant	No activity	No activity
	precipitate	2 minutes	Very strong (titer not determined)
Dialyzed against distilled water	supernatant	15 minutes	4
	precipitate	1 minute	32
Precipitation by adding alcohol		No activity	8
Control sperm extract (prepared by freezing and thawing)		1 minute	64

of these showed high lytic activities of the precipitate as compared to the supernatant (Table 2).

Since a differential precipitation of the lytic factors did not occur, it cannot be concluded on the basis of the above results that there are two different lysins present in the sperm extracts. The presence of two active agents is, however, suggested by a differential effect of heat on the sperm extracts. Table 3 records the results of typical heat tests on a *M. edulis* sperm extract. Samples of the extract were heated at various temperatures for five minute periods and then assayed for the lytic activities. Five minutes at 60° C. was sufficient to inactivate partially the membrane lysin, but had little effect on the cement lysin. Nearly complete inactivation of the membrane lysin occurred within 5 minutes at 80° C.; however, sperm extracts after one hour at 100° C. still caused dissolution of the intercellular cement, but showed no membrane lysis.

TABLE 3

Differential inactivation of lytic activities of a sperm extract by heat

Treatment of sperm extract	Time for dissolution of egg membranes	Titer of cement lysin
5 minutes at 60° C.	5 minutes	64
5 minutes at 80° C.	20 minutes	32
5 minutes at 100° C.	No activity	16 to 32
1 hour at 100° C.	No activity	8
Control	2 minutes	64

The heat stability of the cement dissolving activity of sperm extracts depends in part on the pH of the extract. At pH 3 to pH 8 there is little difference in the rate of inactivation by heat; however, at pH 10 the cement lysin is completely inactivated within a few minutes at 100° C.

Alcoholic precipitation of sperm extracts causes complete inactivation of the membrane lysin and partial reduction of the cement dissolving activity. Chilled ethyl alcohol was slowly added to a sperm extract at 2° C. until a final concentration of 80 per cent was reached. A white precipitate formed which was freed of alcohol and dissolved in sea water. This solution exhibited no membrane-lytic activity; however, its cement dissolving titer was 4 to 8 as compared to 64 for the original extract.

The lytic activities of sperm extracts are destroyed by trypsin. In a typical experiment a trypsin solution was added to a strong sperm extract resulting in a final concentration of 0.1 per cent of the enzyme. After several hours treatment of the extract with trypsin, the extract had no discernible effect on the egg membrane or intercellular cement, indicating that the active agents had been destroyed.

Extracts, prepared by freezing and grinding, of various tissues (other than the testes) of adult mussels exhibited no lytic activities on the eggs.

Sperm extracts may be dried under vacuum without loss of activity, a procedure which has proven useful for obtaining concentrated solutions of the active factors. Concentration of sperm extracts by this method or by precipitation in salt free solutions led to the discovery of an inhibitor of the cement lysin. Although only a low percentage of eggs cleaved in a concentrated extract, the blastomeres of eggs that did cleave were nearly as cohesive as the controls. Assays of the extract showed a high titer of the cement lysin, but its effect was not noticeable until about a $\frac{1}{4}$ to $\frac{1}{8}$ dilution of the original extract. Apparently there was an inhibitor in the extract which over a threshold concentration prevented the dissolution of the cement. Little is known about the nature of this inhibitor except that it does not inhibit the membrane lysin, and that it is destroyed in a few seconds at 100° C.

Species Specificity

Tyler (1939) reported that the membrane lysin from *Megathura* sperm does not dissolve *Haliotis* egg membranes and von Medem (1942, 1945) concluded on the basis of more extensive tests that the membrane lysins of various mollusks are to some extent species specific.

Active sperm extracts are obtainable from both *M. edulis* and *M. californianus* and cross tests were carried out to determine the specificity of the lytic activities. *M. californianus* extracts were able to dissolve the membranes and intercellular cement of *M. edulis* eggs; however, the reverse tests were negative. An *M. edulis* extract had a very weak lytic effect on the *M. californianus* egg membrane and no discernible effect on the intercellular cement.

A few cross tests were carried out on the eggs of other animals. *M. californianus* and *M. edulis* membrane lysins did not dissolve egg membranes of *Acmaea scabra, Mya arenaria, Urechis caupo,* and *Strongylocentrotus purpuratus* nor did sperm extracts of these animals have any effect on their respective egg membranes or the *M. edulis* egg membrane. With the exception of a few experiments, only the membrane-lytic action of the sperm extracts was tested since the membranes of most of the eggs could not be removed for conclusive tests of possible cement lysins. *M. californianus* sperm extracts, however, had no effect on the hyaline layer of sea-urchin eggs after removal of the fertilization membranes.

Discussion

For the purpose of comparison, the main characteristics of the lytic activities of *M. edulis* sperm extracts are summarized in Table 4. It is evident from inspection of this table that the characteristics of these agents closely parallel one another. Similarities exist not only in occurrence and methods of extraction, but also in the chemical behavior. There is no conclusive proof that there are two active agents

present in the sperm extracts although this possibility is strongly suggested by a differential inactivation of the activities by heat. The membrane lysin is extremely heat labile, being partially inactivated in a few minutes at 60° C. and completely inactivated in a short time at higher temperatures. In comparison the cement dissolving activities is relatively heat stable and is only partially inactivated after one hour at 100° C. This greater stability of the cement lysin is also reflected by retention of activity after precipitation in alcohol, whereas the membrane lysin is completely destroyed by this treatment.

TABLE 4

Characteristics of lytic activities of M. edulis sperm extracts

Membrane lysin	Cement lysin
1. Maximum activity obtained by acidification or freezing of sperm suspensions	1. Maximum activity obtained by acidification or freezing of sperm suspensions
2. Not obtainable from other tissues.	2. Not obtainable from other tissues
3. Non-dialyzable	3. Non-dialyzable
4. Inactivated by trypsin	4. Inactivated by trypsin
5. Heat labile	5. Relatively heat stable
6. Precipitated by dialysis against distilled water	6. Precipitated by dialysis against distilled water
7. Inactivated by precipitation in alcohol	7. Partially inactivated by precipitation in alcohol
8. Precipitated in 50% to 90% solutions of $(NH_4)_2SO_4$	8. Precipitated in 50% to 90% solutions of $(NH_4)_2SO_4$
9. Not inhibited in a concentrated sperm extract	9. Inhibited in a concentrated sperm extract

Precipitation by alcohol, $(NH_4)_2SO_4$, and salt free solutions strongly suggests that the active substances are proteins, which, by definition, would be globulins. Inactivation of both the membrane lysin and the cement lysin by trypsin also suggests their protein nature. The enzymatic nature and probable protein structure of the membrane lysin of *Megathura* sperm has been previously pointed out by Tyler (1939) and evidently the membrane lysin of *Mytilus* is similar in nature.

The mechanism of action of the cement lysin is not known. Although it is only slowly inactivated by heat an enzymatic action cannot be completely ruled out. It may act in a way so as to prevent coupling of calcium with the cement protein; however, it is unlikely that this is brought about by binding of free calcium in the sea water. Excess calcium in a sperm extract does not decrease the effectiveness of the extract in causing dissolution of the cement. Also if the mechanism were a binding of free calcium one would not expect a species specificity of sperm extracts. That a species specificity does exist is shown by the fact that *M. edulis* extracts have no effect on *M. californianus* eggs and furthermore they do not dissolve the hyaline layer of membraneless sea-urchin eggs.

Fauré-Fremiet and Thaureaux (1949) have described in detail the action of a number of synthetic detergents on the eggs of *Toredo norvegia*. Separation of the first blastomeres of *Toredo* occurs in weak solutions of detergents in much the same way as a sperm extract separates those of *Mytilus*. Supposedly, detergents are able to interact with and disperse proteins and it may be that the cement dissolving action of a *Mytilus* sperm extract has a similar mechanism of action.

It is unlikely that there is any relation between the cement dissolving activity of *Mytilus* sperm extracts and hyaluronidase of mammalian sperm. The latter is enzymatic in action and acts on the hyaluronate substance of mammalian tissue (reviewed by Meyer, 1947), but has no effect on the intercellular cement of blood capillaries (Chambers and Zweifach, 1947). Also the hyaluronidase-like substance of sea-urchin sperm (Ruffo and Monroy, 1947) has no effect on the intercellular cement of the sea-urchin embryo. It might also be pointed out that beef testis hyaluronidase has no perceptible effect on the membranes and cement of *M. edulis* eggs.

Runnström, et al. (1945, 1946), described a lytic activity of methanol extracts of sea-urchin sperm which apparently caused liquefaction of the egg surface as indicated, in part, by constriction of eggs into fragments at high centrifugal forces. The active agent is heat stable, dialyzable through cellophane, and is not inactivated by trypsin. A few preliminary tests of the cement lysin of *Mytilus* indicated that it did not alter the irregular shapes of unfertilized eggs or facilitate constriction of eggs during severe centrifugation. Thus the egg-surface lysin of sea-urchin sperm and the cement lysin of *Mytilus* show considerable differences in their chemical nature and effects on the eggs.

The function of the several lysins obtainable from the sperm of animals is not clear although possible roles in fertilization have been suggested. A reasonable explanation for the presence of the membrane lysin, as suggested by Tyler (1939), is to aid penetration of the sperm through the egg membrane to the surface of the egg. Although the agent causing dissolution of the intercellular cement may occur in physiological concentrations, it cannot be concluded without further work that it has a role in fertilization.

Summary

Extracts of *Mytilus* sperm contain a lytic substance, or substances, which causes dissolution of the egg membrane and the intercellular cement which binds the blastomeres together. Bioassays were utilized to investigate the occurrence and relationship of the active agents and some of their chemical properties. The lytic effects are exhibited by concentrated sperm suspensions and their supernatants obtained by centrifugation. Extracts prepared by freezing and thawing and by acidification or alkalization exhibit the same relative strengths of lytic activities.

The active agents are large molecules, as indicated by their non-dialyzability through cellophane. They are precipitated by ammonium sulfate, by dialysis against distilled water, and by alcohol. These properties indicate a protein nature of the lytic substances, an assumption which is further supported by the fact that they are inactivated by trypsin.

While there is no conclusive evidence that there are two separately acting lysins, this is strongly suggested, in part, by a differential inactivation of sperm activities by heat. The membrane lysin is heat labile whereas the cement-lytic activity is relatively heat stable. It is concluded that the latter effect is not brought about by a binding of free calcium of the sea water.

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LITERATURE CITED

Berg, W. E., 1949. In press *Am. Nat.*

Bielig, H. J. and G. F. v. Medem, 1949. Wirkstoffe der tierischen Befruchtung. *Experientia,* **5**: 11.

Chambers, R. and B. W. Zweifach, 1947. Intercellular cement and capillary permeability. *Physiol. Rev.,* **27**: 436.

Fauré-Fremiet, E. and J. Thaureaux, 1949. Effect de quelques détergents sur l'oeuf de Teredo norvegica. *Biochim. et Biophy. Acta,* **3**: 536.

Field, I. A., 1921–1922. Biology and economic value of the sea mussel, *Mytilus edulis. Bull. of U. S. Bureau of Fish.,* **XXXVIII**: 127.

Hibbard, H., 1928. Contribution à l'étude de l'ovogenèse, de la fecondation, et de l'histogenèse chez Discoglossus. *Arch. de Biol.,* **38**: 251.

Medem, Graf F. v., 1942. Beiträge sur Frage der Befruchtungsstoffe bei marinen Mullusken. *Biol. Zbl.,* **62**: 431.

Medem, Graf F. v., 1945. Untersuchungen über die Ei und Spermawirkstoffe bei marinen Mollusken. *Zool. Jahr.,* **61**: 1.

Meyer, K., 1947. The biological significance of hyaluronic acid and hyaluronidase. *Physiol. Rev.,* **27**: 335.

Monroy, A. and A. Ruffo, 1947. Hyaluronidase in sea-urchin sperm. *Nature,* **159**: 603.

Monroy, A., 1948. A preliminary approach to the physiology of fertilization in *Pomatoceros triqueter. Arkiv Zool.,* **40**: No. 21.

Ruffo, A. and A. Monroy, 1947. Ricerche sulla fisiologia della fecondazione. *Pubbl. Staz. Zool., Napoli,* **20**: 253.

Runnström, J., A. Tiselius and S. Lindvall, 1945. The action of adrogamone III on the sea-urchin egg. *Arkiv Zool.,* **36A**: 1.

Runnström, J. and S. Lindvall, 1946. The effect of some agents upon the reaction of Echinocardium spermatozoa towards egg-water. *Arkiv Zool.,* **38A**.

Tyler, A., 1939. Extraction of an egg membrane-lysin from sperm of the giant keyhole limpet (*Megathura crenulata*). *Nat. Acad. of Sci.,* **25**: 317.

Tyler, A., 1948. Fertilization and immunity. *Physiol. Rev.,* **28**: 180.

Wintrebert, P., 1929. La digestion de l'enveloppe tubaire interne de l'oeuf par des ferments issus des spermatozoides, et de l'ovule, chez Discoglossus. *Compt. Rend. Acad. Sci.,* **188**: 97.

Wintrebert, P., 1933. La fonction enzymatique de l'acrosome spermien du Discoglosse. *Compt. Rend. Soc. Biol.,* **112**: 1636.